Published in final edited form as:

Fertil Steril. 2011 August ; 96(2): 474–478.e4. doi:10.1016/j.fertnstert.2011.05.017.

Reduced Neurotrophin Receptor TrkA Expression in Human Granulosa Cells: A Novel Marker of Diminishing Ovarian Reserve

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Structured Abstract

Objective—To determine whether human mural and cumulus granulosa cell neurotrophin and neurotrophin receptor content correlate to ovarian reserve markers.

Design—Prospective, laboratory based study

Setting—Academic assisted reproductive technology (ART) program

Patients—Twenty three women undergoing ART

Interventions—Mural and cumulus granulosa cells were collected from women undergoing oocyte retrieval during ART cycle. Relative mRNA levels of neurotrophins and their receptors were measured by qRT-PCR and correlated to serum anti-mullerian hormone (AMH) levels and the number of oocytes retrieved.

Results—Mural and cumulus granulosa cell nerve growth factor receptor TrkA mRNA correlated strongly to the number of oocytes retrieved. Similarly, higher serum AMH was associated with higher cumulus granulosa cell TrkA mRNA. Both mural and cumulus granulosa cell p75^{NTR}/TrkA ratios were lower in women with higher serum AMH and the number of oocytes retrieved were greater among women with low p75^{NTR}/TrkA ratio. No significant associations were found between brain derived neurotrophic factor (BDNF) and its specific receptor TrkB and ovarian reserve markers. While BDNF and TrkB expression were higher in cumulus compared to mural granulosa cells, no such association was found between TrkA and

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None of the authors have any conflict of interest to disclose.

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granulosa cells. AMH and cumulus TrkA mRNA, in a model incorporating both, correlated strongly to the number of oocytes retrieved ($R^2=0.84$, $p=0.001$).

Conclusions—Cumulus TrkA and p75^{NTR} mRNA correlate to ovarian reserve while BDNF and TrkB are associated with the type of granulosa cell.

Introduction

Neurotrophins (NTs) are nerve growth factors important in the development of the central and peripheral nervous systems (1). Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3, and NT-4/5 are major NTs and bind two kinds of receptors. p75^{NTR} is a member of the tumor necrosis factor receptor super family and binds all NTs with equal but low affinity. It promotes survival or apoptosis of the cell, depending on TrkA receptor content. Cells that have high p75^{NTR}/TrkA ratio undergo apoptosis in response to NGF or proNGF, whereas those cells with a low ratio respond with survival, in most cellular contexts (2). Each neurotrophin also has its own specific tyrosine kinase receptor: NGF specifically binds and activates TrkA; BDNF and NT-4, TrkB; and NT-3, TrkC. NTs, together with their receptors, are found in animal and human ovaries (3) (4) (5) and have a wide variety of ovarian functions, including control of steroidogenesis by NGF (6), extrusion of the oocyte polar body by NT-4 (7), and ovulation (8). They also play a crucial role in ovarian folliculogenesis as evidenced by knockout mice models. NGF^{-/-}, NT-4^{-/-}, and BDNF^{-/-} mice have decreased numbers of primary and secondary ovarian follicles (9, 10); additionally, TrkB^{-/-} mice have greatly reduced numbers of follicles (11) many of which are arrested at the primary stage of development (12).

Neurotrophins, especially NGF, are associated with brain aging. They support the survival of cholinergic neurons and prevent their age-dependent loss (13). Age-related decline in the number of TrkA receptors and the resulting switch from TrkA to low affinity receptor p75^{NTR} predominance may mediate the cholinergic neuron dysfunction leading to cognitive loss (14, 15). Estrogen and NT receptors are widely co-localized in the brain, resulting in reciprocal transcriptional regulation by their respective ligands and cross-coupling of their signaling pathways (16). Thus, estrogen, together with NTs, may act as growth factors important in development, survival and aging of the mammalian brain.

By the same token, NTs may also support the survival and growth of ovarian follicles. We have previously demonstrated differential secretion of NTs into ovarian follicular fluid in infertile women undergoing treatment with assisted reproductive technology (ART). Women with signs of premature ovarian aging and diminished ovarian reserve (DOR) have significantly higher levels of follicular fluid NGF levels compared to women with male factor infertility (control group) (17). DOR is a condition associated with both decreased number and quality of oocytes retrieved during an ART cycle, as evidenced by decreased clinical pregnancy rates (18, 19). DOR is the result of the normal aging process. However it may also result from genetic, autoimmune or iatrogenic conditions or it may be idiopathic. Genetically, women with the FMR1 mutation have a 20-30% chance of suffering from this condition (20). In addition, autoimmune endocrinopathies, radiation therapy or pelvic surgery may also lead to DOR (19, 21, 22). In clinical practice, DOR is most often diagnosed by elevated serum follicle stimulating hormone (FSH) levels on day 2 or 3 of menstrual cycle (baseline). However, an antral follicle count less than 5 per ovary, low ovarian volume, low inhibin B levels, less than 5 oocytes retrieved during an ART cycle and recently, low anti-mullerian hormone (AMH) levels, are all used to define DOR (3).

Anti-Mullerian hormone (AMH) is a glycoprotein hormone that belongs to TGF- β family. It is the earliest marker of ovarian reserve to change with age (23) and one of the best predictors of the number of oocytes retrieved (24) and, recently, the pregnancy outcome

during *in vitro* fertilization (IVF) cycles (25). It is secreted from preantral and early antral follicles and detected in the serum. The serum levels are relatively stable during the menstrual cycle, unlike other markers of ovarian reserve, like FSH.

We hypothesized that NTs and their receptors would demonstrate aging-related changes similar to those seen in brain when examined in the ovarian granulosa cells of women with DOR. We examined NT and NT receptor gene expression profiles in both mural and cumulus granulosa cells obtained from women with and without DOR, who underwent IVF. We correlated the relative mRNA levels of granulosa cell NTs and their receptors to serum anti-Mullerian hormone (AMH) levels and the number of oocytes retrieved during the IVF cycle to elucidate the association between NTs/NT receptors and ovarian reserve.

Materials and Methods

See supplemental material.

Results

Baseline characteristics of women participating to the study are shown in Supplemental Table 1. Because we included patients with normal ovarian reserve (NOR) as well as DOR, age (35; 27-46), maximum baseline FSH (9; 4-35), AMH (1.3; 0.25-9.55) and the number of eggs retrieved (13; 5-31) had a wide range of distribution (median and range, respectively).

Granulosa cell TrkA mRNA levels correlate significantly with the markers of ovarian reserve

The association between mural and cumulus granulosa cell TrkA mRNA levels and number of oocytes retrieved is shown in Figure 1. In both mural and cumulus cells, a statistically significant association was observed between the TrkA mRNA level and the number of oocytes retrieved ($r=0.62$, $p=0.008$ and $r=0.86$, $p=0.0001$, respectively).

Out of twenty three study participants, fifteen had their serum AMH levels measured before the start of their ART cycles and their baseline and cycle characteristics according to their AMH levels are shown in Table 1. No statistically significant difference was seen between the two groups except their serum AMH levels, however, the sample size was too small to draw any conclusions. We observed a statistically significant correlation between serum AMH levels and the number of oocytes retrieved ($r=0.6$, $p=0.02$) (Figure 2a). When women were grouped according to their serum AMH levels, those women with an AMH cutoff ≥ 0.8 ng/ml had a non-significant trend towards increased TrkA mRNA in their mural granulosa cells (8.8 ± 1.3 vs. 4.5 ± 1.3 , $p=0.06$) and significantly more TrkA mRNA in their cumulus granulosa cells (10.7 ± 1.3 vs. 4.7 ± 3.1 , $p=0.03$) (Figure 2b, c).

Granulosa cell p75NTR/TrkA ratio correlates significantly with the markers of ovarian reserve

Low affinity NT receptor p75^{NTR} is a member of the TNF α super-family and promotes survival or apoptosis of the cell, depending on TrkA receptor content. Cells that have high p75NTR/TrkA ratio undergo apoptosis in response to NGF or proNGF, whereas those cells with a low ratio respond with survival, in most cellular contexts (2). We found that those women with an AMH level of 0.8 ng/ml or above had a lower p75NTR/TrkA ratio in both mural and cumulus granulosa cells, compared to those women who had an AMH level below 0.8 ng/ml (1.9 ± 0.4 vs. 4.5 ± 1.3 , $p=0.05$ in mural cells, and 1.5 ± 0.2 vs. 7.4 ± 4 , $p=0.02$ in cumulus cells) (Supplemental Figure 1a, b). Moreover, women with a p75NTR/TrkA ratio above 2 in their cumulus granulosa cells were more likely to have fewer oocytes retrieved than those with a lower ratio (6.8 ± 0.9 vs. 17 ± 2.4 , $p=0.02$) (Supplemental Figure 1c).

Interestingly, the same association was not found with mural granulosa cells (data not shown).

Granulosa cell NGF, BDNF and TrkB mRNA levels do not significantly correlate with ovarian reserve markers

Next, we measured the relative mRNA levels of NGF, BDNF and TrkB, which is the specific tyrosine kinase receptor of BDNF. There was no significant association between the mRNA levels of NGF, BDNF and TrkB and AMH nor the number of oocytes retrieved, suggesting that none of these genes are associated with ovarian reserve (data not shown).

BDNF and its specific receptor TrkB are differentially expressed in granulosa cells

We measured the relative mRNA levels of BDNF and TrkB in mural and cumulus granulosa cells. Although this NT and its receptor are not associated with ovarian reserve markers, we found that BDNF mRNA expression was almost twofold higher in cumulus vs. mural granulosa cells (6.4 ± 0.9 vs. 3.5 ± 0.5 , $p=0.004$). Similarly, its receptor TrkB mRNA expression was almost threefold higher in cumulus vs. mural granulosa cells (71.6 ± 8.9 vs. 25.4 ± 4.5 , $p<0.0001$) (Supplemental Figure 2a, b). Conversely, while TrkA and p75^{NTR}/TrkA ratio were associated with ovarian reserve markers, their mRNA levels were similar in both types of granulosa cells. NGF mRNA expression was also similar in both cell types (data not shown).

Cumulus TrkA mRNA expression is an independent predictor of ovarian reserve

We created a model that associates with the number of oocytes retrieved during the ART cycle. Using linear regression analysis adjusting for confounding variables serum AMH and cumulus TrkA mRNA levels, the model predicts the number of oocytes retrieved with an adjusted R^2 of 0.84 and a p value of 0.001 (Figure 3). Addition of cumulus TrkA mRNA level to AMH increased the adjusted R^2 value from 0.52 (AMH only) to 0.84 (AMH and cumulus TrkA mRNA).

Discussion

Herein we report an association between granulosa cell TrkA receptor content and ovarian reserve. It has been shown that NGF and its receptor, TrkA, support the survival of cholinergic neurons in the brain and that treatment with anti-NGF monoclonal antibodies and TrkA receptor antagonists lead to loss of cholinergic bouton numbers in nucleus basalis (13). We found a similar association in the human ovary, where the levels of TrkA mRNA correlate closely with markers of ovarian reserve, mainly AMH and the number of oocytes retrieved during an ART cycle. It is possible that, similar to its role in the brain, TrkA mediates survival signals, especially in cumulus granulosa cells, necessary to maintain a healthy cumulus-oocyte complex. Hence, a decrease in cumulus cell TrkA receptor levels, either due to aging or some other yet unknown factor, may eventually lead to the loss of survival signals to the oocyte and ultimately to diminished reserve in the ovary. This may explain, in part, the occurrence of DOR in young women and the predictability of ovarian reserve by TrkA levels.

A switch from TrkA to p75^{NTR} predominance is one of the characteristics of the aging brain (14). It has been previously shown that the unprimed PC12 cells (pheochromocytoma cell line) have a higher p75^{NTR}/TrkA ratio (1.7) than primed PC12 cells (0.4). When unprimed PC12 cells are stimulated with proNGF (the propeptide for NGF), they undergo apoptosis whereas stimulation of primed PC12 cells supports survival (2). Hence, the ratio of p75^{NTR}/TrkA in a given cell determines the fate of the cell. We observed a significant negative association between the markers of ovarian reserve (AMH and the number of oocytes

retrieved) and the $p75^{NTR}/TrkA$ ratio in cumulus cells. A high AMH level is associated with low $p75^{NTR}/TrkA$ ratio. Similarly, more oocytes are retrieved when this ratio is below 2. Since a high ratio is detrimental to the cell in terms of survival, we postulate that the acquisition of a high $p75^{NTR}/TrkA$ ratio phenotype is detrimental to the cumulus-oocyte complex and is associated with DOR. Moreover, high NGF levels in the follicular fluid of women with DOR (17) may actually worsen the quality of the cumulus-oocyte complex by activating the $p75^{NTR}$ -JNK pathway and lead to a DOR phenotype.

These findings suggest that there is a close association, not yet demonstrated to be causal, between the receptors TrkA and $p75^{NTR}$, and ovarian reserve, with a high $p75^{NTR}/TrkA$ ratio in granulosa cells favoring DOR. One interesting finding of our study is that cumulus granulosa cell NT receptor content is more closely related to markers of ovarian reserve than mural granulosa cell receptor content. One possible explanation is that the collection of mural cells is more cumbersome, with possible contamination from theca cells and other ovarian stromal cells while cumulus cells are collected under direct visualization using a microscope and are thus less likely to be contaminated. However it is also possible that due to their juxtaposition to the oocyte, cumulus cells may be more important than mural cells in determining the survival and quality of the oocyte.

While TrkA and $p75^{NTR}$ correlate significantly with the ovarian reserve markers, their mRNA levels are similar in both mural and cumulus granulosa cells. On the other hand, there was no evidence of association between BDNF and its receptor TrkB and ovarian reserve markers, but their levels are increased significantly in cumulus compared to mural granulosa cells. It is possible that BDNF and TrkB are associated with morphologic characteristics of granulosa cells while the balance between TrkA and $p75^{NTR}$ determines the survival capacity of the cumulus-oocyte complex, and hence determination of DOR characteristics. Although highly speculative, it is possible that with age, or other yet unknown environmental factors, a switch from TrkA to $p75^{NTR}$ receptor predominance in cumulus granulosa cells may occur in women with DOR.

Although we observed significant associations between NT receptors and ovarian reserve markers in this prospective study, it is limited by its relatively small sample size. Moreover, although these receptors may be markers of ovarian reserve, a causal relationship has not been established. We are also limited by the fact that the luteinized granulosa cells are collected from women who are hyperstimulated with gonadotropins, and hence present less physiologic model. On the other hand, the stimulation protocols were similar for all women and the differential gene expression seen under these supraphysiologic conditions may point to a bigger difference in the physiologic state.

In summary, we report for the first time that NT receptors TrkA and $p75^{NTR}$ are associated with ovarian reserve. We also show that BDNF and its receptor TrkB are differentially expressed in mural and cumulus granulosa cells, and that they are not predictive of ovarian reserve. These results are consistent with our hypothesis that neurotrophins and their receptors play important and differential roles in multiple aspects of ovarian function. More studies are needed and some are underway to decipher the pathogenesis of TrkA/ $p75^{NTR}$ -associated loss of ovarian function and the differential expression of BDNF/TrkB in mural and cumulus granulosa cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported in part by a Grant from Resnick Gerontology Center of Albert Einstein College of Medicine to EB; by a Grant from Ferring Pharmaceuticals to EB and SJ; by Center for the Study of Reproductive Biology and Women's Health of Albert Einstein College of Medicine and by the CTSA Grant UL1 RR025750, KL2 RR025749 and TL1 RR025748 to HC from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH), and NIH roadmap for Medical Research. Its contents are solely the responsibility of the authors and do not necessary represent the official view of the NCRR or NIH.

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Figure 1. Granulosa cell TrkA mRNA levels correlate significantly with the number of oocytes retrieved during an ART cycle. Mural (A) and cumulus (B) TrkA mRNA levels are significantly associated with the number of oocytes retrieved ($p=0.008$, and $p=0.0001$, respectively).

Figure 2.

Granulosa cell TrkA mRNA levels correlate significantly with serum AMH levels measured at the start of an ART cycle. A) AMH correlates closely with the number of oocytes retrieved ($r=0.73$, $p=0.002$). B,C) Mural and cumulus TrkA mRNA level is higher in women with high serum AMH levels ($p=0.06$ and $p=0.03$, respectively).

Figure 3.

Linear regression analysis adjusting for serum AMH and cumulus TrkA mRNA levels shows that the model including AMH and cumulus TrkA mRNA level is strongly correlated to the number of oocytes retrieved during an ART cycle ($p=0.001$, $R^2=0.84$).

Table 1
Baseline and cycle characteristics of women according to their AMH levels

Number of women	AMH<0.8 n=3	AMH≥0.8 n=12	P
Age (years)	40±4.4	35.8±5	0.1
BMI (kg/m ²)	24.8±1.9	26±4.9	0.6
Maximum historical FSH (mIU/ml)	10.7±1.2	10.4±8.1	0.2
AMH (ng/ml)	0.4±0.09	2.3±2.4	0.009
Controlled ovarian hyperstimulation			
Recombinant FSH (n,%)	0 (0)	8 (67)	0.08
Recombinant FSH + HMG (n,%)	3 (100)	4 (33)	0.08
GnRH Agonist Suppression (n,%)	2 (67)	10 (83)	0.5
GnRH Antagonist Suppression (n,%)	1 (33)	2 (17)	0.5
Number of oocytes retrieved	8.3±4.9	16.3±7.3	0.08

mean±SD